

### Process for the purification of recombinant polypeptides

This invention is concerned with a method for preparation of a recombinant polypeptide of interest, which polypeptide upon expression has been secreted into the periplasm of a transformed host cell.

In particular, this invention is concerned with methods of preparing and purifying a recombinant human interferon alpha 2.

Polypeptides or proteins, like interferons of the group of interferon alpha 2, may be produced by recombinant DNA technology using bacterial cells (e.g. *Escherichia coli*) as hosts. Thus, bacterial cells may be transformed with plasmid DNA encoding said polypeptide. The bacteria are thereby enabled to express quantities of the polypeptide in either the cytoplasm or the periplasm. As the bacteria can be grown in large amounts using large-scale fermentation processes, it is possible to produce large quantities of the polypeptide in this way.

Whereas recombinant techniques can be employed to produce high yields of a crude polypeptide of interest, the isolation and purification of the polypeptide is not a simple matter. In a typical isolation procedure, a fermentation broth is neutralised, for example by acidification or heating. Thereafter, the bacterial cells are removed to leave a liquid supernatant, containing unwanted soluble by-products, which is discarded. The resultant bacterial cell mass is re-suspended in an appropriate medium, e.g. a suitable buffer and the cells are disrupted to extract and isolate the crude interferon. This laborious procedure is carried out in order to separate the polypeptide of interest from as much fermentation by-products and other contaminants as possible to ensure that subsequent purification steps (involving chromatographic separation) proceed in as an efficient manner as possible.

The laborious nature of this prior art procedure may lead to lower yields of extracted polypeptide of interest and higher production costs. Accordingly, there remains a need for a process that enables the preparation of a recombinant polypeptide of interest from bacterial cells in a high yielding and cost-effective manner.

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In the context of the present invention it has surprisingly been found that efficient extraction and isolation of a recombinant polypeptide of interest, for example of a recombinant interferon alpha 2, from a host cell comprising a periplasm, like a Gram-negative bacterial cell, is possible by directly performing an osmotic shock on the host cells comprising an expressed recombinant polypeptide of interest in their periplasm, thereby omitting the aforementioned separation and re-suspension steps. Upon performance of such a process the outer cell membrane of the host cell is sufficiently disrupted as to release the contents of its periplasm into the fermentation medium. Thereby, the release of unwanted cytoplasmic material, e.g. host-cell proteins and DNA in the cell debris fraction, can be avoided. Surprisingly, subsequent chromatographic purification is not compromised, but readily leads to superior yields and/or purity of the recombinant polypeptide to be isolated.

In the context of the present invention it has furthermore been found that a unique sequence of chromatographic steps, in particular if combined with the extraction / disruption process mentioned above, leads to superior yields and/or purity of a recombinant interferon alpha 2.

Accordingly, in one aspect of the present invention, there is provided a process for the preparation of a recombinant polypeptide of interest, comprising

- (i) fermentation of a prokaryotic host cell comprising a periplasm and being transformed with a recombinant expression system capable of bringing about secretion of a polypeptide of interest into the periplasm of said host cell, wherein said fermentation is performed in a fermentation medium under conditions such that the polypeptide of interest is secreted into the periplasm of the host cell,
- (ii) extraction of the polypeptide of interest from the periplasm by applying an osmotic shock to the host cells contained in the fermentation medium.

Suitable recombinant periplasmic expression systems, in particular expression vectors, and corresponding prokaryotic host cells as well as appropriate fermentations methods are well known in the art. Suitable examples are described below in the Examples section.

In a preferred embodiment thereof said osmotic shock is performed by adding an agent directly to the fermentation medium, wherein said agent is capable of creating after dilution with H<sub>2</sub>O an osmotic pressure leading to disruption of the outer cell membrane of the host cell, and subsequent dilution with H<sub>2</sub>O.

Preferably, the agent is selected from the group consisting of sucrose, sodium chloride, arginine, lysine, guanidine hydrochloride, Triton-X 100, polyethyleneimine, and suitable mixtures thereof, i.e. mixtures of two or more of such agents. Most preferably, said agent is sucrose. Optionally, a complex forming component, like EDTA, may additionally be added to the fermentation medium.

The agent is present in such a concentration as, upon dilution of the fermentation medium with H<sub>2</sub>O, to bring about an osmotic shock which leads to disruption of outer cell membrane of the host cell with subsequent release of the expressed polypeptide of interest.

In particular, in a further preferred embodiment of the present invention, the concentration of the sucrose in the fermentation medium when starting the dilution is about 20% weight/volume. Preferably, the dilution factor of the sucrose-containing fermentation broth with H<sub>2</sub>O is at least about 3 times.

In the context of the present invention, a preferred prokaryotic host cell comprising a periplasm is a Gram-negative bacterium. Preferably, the said Gram-negative bacterium is selected from the group consisting of *Escherichia coli*, *Pseudomonas* sp., *Enterobacter* sp., *Campylobacter* sp. and *Vitreoscilla* sp. In a most preferred embodiment of the present invention, the host cell is *E. coli*.

Subsequent to the cell disruption step, the fermentation broth, being a crude preparation of the recombinant polypeptide of interest, may be subjected to a separation step, e.g. high speed centrifugation, in order that cellular debris and other particulate matter can be separated from the extract containing the polypeptide of interest. To assist in the separation of particulate matter from the extract it is preferred to add to the fermentation broth prior to the separation step, a suitable precipitating agent. In the context of the present invention it has been found that polyethyleneimine is a particularly good precipitating agent for this step. The polyethyleneimine is preferably employed at a concentration of about 0.05% and in a medium which is pH adjusted to about 7.5, e.g. 7.3 to 7.7.

The process of the present invention can be utilized in the production of a large variety of polypeptides of interest. In particular, in accordance with the present invention, the

polypeptide of interest can be selected from the group consisting of an interferon, an interleukin, a growth hormone, a growth factor, a cytokine, an enzyme, an enzyme inhibitor, an antibody and an antibody fragment, and the like, for example interferon alpha 2A, interferon alpha 2B, interleukin-3, interleukin-6, human growth hormone, insulin, granulocyte-colony stimulating factor, granulocyte macrophage-colony stimulating factor, macrophage-colony stimulating factor, interferon beta 1, bovine somatotropin, porcine somatotropin, interleukin-11, interleukin-2, a Fab-fragment, and small peptides such as calcitonin, parathyroid hormone (PTH), or a glucagon. Preferably, within the scope of the present invention, the polypeptide of interest is a recombinant human interferon 2, in particular human interferon alpha 2A or human interferon alpha 2B, the latter being particularly preferred to be the polypeptide of interest.

The extract comprising the polypeptide of interest may contain a number of impurities, for example host-cell proteins and host-cell DNA which have to be removed before the interferon can be formulated into a finished dosage form. Typically, the polypeptide is purified by precipitation and chromatographic separation techniques, which are known per se. For example, the polypeptide may be purified using multi-step chromatographic separation.

However, depending on the nature of the polypeptide of interest, the process is complicated by the need for significant dialysis and/or concentration steps interposed between the chromatographic steps. These extra steps are laborious and may lead to lower yields and higher production costs.

In the context of the present invention it has been found that a multi-step chromatographic purification of a crude preparation, in particular prepared as described above, of recombinant interferon alpha 2, may be carried out without any dialysis or concentration steps by the judicious selection and ordering of certain chromatographic steps.

Therefore, another aspect of the present invention relates to a process for the preparation of a recombinant interferon alpha 2, comprising

- (a) obtaining a crude preparation of a recombinant interferon alpha 2,
- (b) applying the crude preparation to a multi-step chromatography comprising the following steps in sequence:

- (i) cation exchange chromatography,
- (ii) anion exchange chromatography,
- (iii) hydrophobic interaction chromatography,
- (iv) cation exchange chromatography,
- (v) size exclusion chromatography.

In a preferred embodiment thereof, the crude preparation of the recombinant interferon alpha 2 is obtained by a process comprising

- (a) fermentation of a prokaryotic host cell comprising a periplasm and being transformed with a recombinant expression system capable of bringing about secretion of a recombinant interferon alpha 2 into the periplasm of said host cell, wherein said fermentation is performed in a fermentation medium under conditions such that the recombinant interferon alpha 2 is secreted into the periplasm,
- (b) extraction of the recombinant interferon alpha 2 from the periplasm by applying an osmotic shock to the host cells contained in the fermentation medium.

As mentioned herein, suitable recombinant periplasmic expression systems, in particular expression vectors, and corresponding prokaryotic host cells as well as appropriate fermentations methods are well known in the art. Suitable examples are described below in the Examples section.

Preferably and as mentioned above, said osmotic shock is performed by adding an agent directly to the fermentation medium, wherein said agent is capable of creating after dilution with H<sub>2</sub>O an osmotic pressure leading to disruption of the outer cell membrane of the host cell, and subsequent dilution with H<sub>2</sub>O.

In a preferred embodiment of the present invention, the agent is selected from the group consisting of sucrose, sodium chloride, arginine, lysine, guanidine hydrochloride, Triton-X 100, polyethyleneimine and suitable mixtures thereof, i.e. mixtures of two or more of such agents. Most preferably, said agent is sucrose. Optionally, a complex forming component, like EDTA, may additionally be added to the fermentation medium.

As mentioned herein, the agent is present in such a concentration as, upon dilution of the fermentation medium with H<sub>2</sub>O, to bring about an osmotic shock which leads to disruption of

outer cell membrane of the host cell with subsequent release of the expressed polypeptide of interest.

Preferably, the concentration of the sucrose in the fermentation medium when starting the dilution is about 20% weight/volume. In a preferred embodiment thereof, the dilution factor for the sucrose-containing fermentation broth with H<sub>2</sub>O is at least about 3 times.

As mentioned herein, a preferred prokaryotic host cell is a Gram-negative bacterium, which preferably is selected from the group consisting of *Escherichia coli*, *Pseudomonas* sp., *Enterobacter* sp., *Campylobacter* sp. and *Vitreoscilla* sp., *E. coli* being particularly preferred.

Said interferon alpha 2 preferably is selected from the group consisting of interferon alpha 2A and interferon alpha 2B. In a most preferred embodiment, said interferon alpha 2 is interferon alpha 2B.

In the extraction method hereinabove described it is possible to obtain a clear crude extract containing the recombinant interferon alpha 2. The polypeptide-containing extract will have a high content of the interferon alpha 2 in highly purified form.

In carrying out the cation exchange chromatography (CEX) step, the pH of the crude interferon alpha 2-containing extract obtained from the extraction step may be adjusted to a pH of about 4.8 to 5.5 and may optionally be passed through a filter system (e.g. 0.3 micrometre filter system). The treated extract is thereafter eluted on a CEX column. Any cation-exchange column known in the art may be useful for separating the extracted interferon alpha 2 from impurities. Preferably however, the column is packed with S ceramic Hyper D F. The equilibration eluent is preferably sodium acetate (20mM) + NaCl (70mM) at a pH of 5.0. The interferon is run on the column preferably using a step gradient at 175mM NaCl.

The pH of the desired interferon alpha 2-containing fraction eluted from the CEX column may be adjusted to about 7.3. to 7.7 with an appropriate alkaline material, e.g. sodium hydroxide, and the conductivity of the fraction may be adjusted to about 3.5 to 4.5 mS/cm by dilution using purified water.

Thereafter, the fraction is fed onto an anion-exchange column to effect the process of step ii). Any anion exchange column known in the art may be useful for separating the extracted interferon from impurities. Preferably however, the column is packed with Ceramic Q HyperD F resin. The equilibration is preferably 20mM Tris-HCl at pH 7. and thus at high flow rates, e.g. 4 - 8 cm/min. The desired interferon fraction may be eluted after washing the column with equilibration buffer by adding a suitable ionic solute, e.g. sodium chloride at a concentration of up to 1000 mM, preferably 150mM. The temperature at which the separation is run is preferably from 10°C to 15°C.

This anion exchange step is highly efficient and the purity of the interferon in the interferon fraction resultant from step ii) may be greater than 40% as determined by reverse-phase high performance liquid chromatography.

After the completion of step ii) the interferon fraction may still be contaminated with host-cell proteins. Accordingly, step iii) in the purification process is a Hydrophobic Interaction Chromatography (HIC) step and is adapted to remove, *inter alia* substantial amounts of these proteins. The step is carried out on a column packed with a suitable resin for this purpose. Preferably the resin is 15PHE (Pharmacia). In a preferred step iii) the interferon fraction to be eluted on the column is first diluted (1:1) with a sodium sulphate solution to a concentration of 0.5M sodium sulphate. Thereafter, the fraction is pH adjusted to about 7.3 to 7.7 with a suitable acid or base, e.g. NaOH or HCl. This pH adjusted solution is then added to the HIC column. After a washing step, the interferon is eluted with a linear sodium sulphate gradient, preferably 800 to 0mM sodium sulphate. Fractions are collected and pooled that have a purity of greater than or equal to 93 area % interferon and no single impurity having greater than or equal to 3 area % according to IPC reversed-phase HPLC. The pooled fractions may be used immediately in the next step.

Step iv) is a further cation exchange chromatography (CEX) step which serves to remove residual DNA and residual host-cell proteins which may have been carried over from previous steps.

A CEX column is packed with a suitable packing material, e.g. Toyopearl SP-650 S (TosoHaas). In a preferred step iv) the fraction obtained from step iii) may be diluted with purified water to a final conductivity of about 7.5 to 8.5 mS/cm, adjusted to a pH of 4.3 to 4.7

with, for example with 99-100% acetic acid, and applied to the column. The column is washed and thereafter interferon alpha 2 is eluted during a linear sodium chloride gradient (0-300mM NaCl) at about 250mM NaCl. Eluted fractions having a purity of greater than or equal to 95 area% interferon main peak and no single impurity greater than or equal to 3 area % as measured by IPC reversed-phase HPLC may be collected and processed immediately in the next purification step.

Step v) is a size exclusion chromatography step which is employed to remove dimers and any other aggregates and, where applicable, also to perform a buffer change which may be necessary before the interferon product can be formulated into a finished dosage form.

Any column and packing material suitable for gel filtration may be employed for this final step. Preferably the packing material employed is Superdex 75pg. The packing material is chosen for its good resolution capabilities even at relatively high load volumes of, for example about 5 to 15%. Preferably the column is equilibrated with an equilibration buffer before being loaded with the interferon fraction from the previous step iv). Thereafter the interferon fraction is eluted off the column using a suitable buffer which preferably consists of 25 mM sodium phosphate, 130 mM sodium chloride and 0.3 mM EDTA at a pH of 7.1 - 7.7 to provide a final bulk solution containing the interferon alpha 2 product.

The process as hereinabove described constitutes an efficient process of obtaining an interferon alpha 2 product which is applicable on an industrial scale. It is possible to obtain yields of the interferon product exceeding 100 mg per liter of fermentation broth.

The interferon alpha 2 polypeptide of the present invention may be formulated into finished dosage forms suitable for administering to humans. Formulations containing interferon alpha 2 products of the present invention may be formulated as injectable formulations. Injectable formulations may be provided as lyophilised products that should be reconstituted with water before administration. Alternatively, injectable formulations may be provided as injectable for solutions as single or multidose preparations. Injectable formulations may additionally comprise excipients commonly known in the art.

Such formulations are useful in the treatment of Hepatitis C. The dosage may depend on the various factors such as the method of administration, age and/or individual condition.



The following examples serve to illustrate the present invention, without in any way limiting the scope thereof. Subject-matter disclosed in the examples relates to preferred embodiments of the present invention.

### Examples

Example 1: Construction of a host cell strain for production of recombinant human interferon alpha 2B (rhIFN $\alpha$ 2B)

#### 1.1 General considerations

The polypeptide rhIFN $\alpha$ 2b (recombinant human Interferon- $\alpha$ 2b) is produced in the *Escherichia coli* K-12 strain W3110 transformed with a plasmid containing an optimized synthetic gene coding for rhIFN $\alpha$ 2b. rhIFN $\alpha$ 2b is produced under the control of the promoter and Ribosome Binding Site (RBS) of the glutaryl 7-ACA acylase gene (*gac*) from *Pseudomonas diminuta* CCM 3987 by fermentation of recombinant *E. coli* K-12. rhIFN $\alpha$ 2b is expressed as an N-terminal fusion protein with the signal sequence from the same (*gac*) gene, directing the protein to the periplasm with concurrent processing (cleaving off) of the signal sequence. The fermentation process therefore directly yields mature rhIFN $\alpha$ 2b with a primary sequence identical to that of naturally occurring human Interferon alpha 2b. The expression plasmid is designated pMG414, the production strain W3110[pMG414].

#### 1.2 Construction of expression vector pMG414

pUC19 serves as the starting point for the construction of the vector plasmid. pUC19 is a frequently used and thoroughly characterized high copy plasmid. It contains a highly efficient origin of replication and an ampicillin resistance (*amp* or *bla*) gene (Yanisch-Perron *et al.*, 1985; Vieira and Messing, 1982; GenBank accession numbers L09137 and X02514). Even though pUC19 is frequently used for the construction of expression plasmids, the *amp* gene may not be an ideal selectable marker for industrial purposes. For this reason the promoter and the coding region of the *amp* gene are removed and replaced by the promoter and the coding region of the tetracycline resistance gene (*tet*) from the well known safety plasmid pBR322 (Bolivar *et al.*, 1977a, 1977b, 1978; review: Balbás *et al.*, 1986; GenBank

accession numbers J01749, K00005, L08654, M10283, M10286, M10356, M10784, M10785, M10786, M33694, V01119). This cloning work is performed with the help of high fidelity PCR techniques.

To achieve this, the fragment spanning bps 1743 to 679 of pUC19 is amplified using high fidelity PCR (Pwo DNA Polymerase system from Roche Biochemicals) and the following 5'-phosphorylated oligonucleotides:

Oligo 235: 5'- Phosphate - TAACTGTCAG ACCAAGTTTA CTC -3' (SEQ ID NO 1)

Oligo 236: 5'- Phosphate - GCGTTTCGGT GATGACGGTG -3' (SEQ ID NO 2)

The resulting PCR fragment is 1624 bps in length and contains the complete pUC19 backbone lacking the *amp* promoter and coding sequence, but including the stop codon and transcription terminator from the *amp* gene.

As mentioned above, the *tet* promoter and coding sequence (excluding the stop codon) is amplified from pBR322. Again, high fidelity PCR was used to amplify bps 4 to 1273 of pBR322. The following 5'-phosphorylated oligonucleotides were used for this amplification:

Oligo 237: 5'- Phosphate - TCATGTTTGA CAGCTTATCA TCG -3' (SEQ ID NO 3)

Oligo 238: 5'- Phosphate - GGTGAGGTG GCCCGGCTC -3' (SEQ ID NO 4)

The resulting PCR fragment is 1270 bps in length. The two PCR fragments are purified by preparative agarose gel electrophoresis and ligated using T4 DNA Ligase (Rapid DNA Ligation Kit, Roche Biochemicals). The ligated DNA is purified and electroporated into *Escherichia coli* K-12 DH10B (Life Technologies ElectroMAX DH10B electrocompetent cells, genotype: F<sup>-</sup> *mcrA*  $\Delta$ (*mrr-hsdRMS-mcrBS*)  $\phi$ 80d/*lacZ*  $\Delta$ M15  $\Delta$ *lacX74* *deoR* *recA1* *endA1* *araD139*  $\Delta$ (*ara, leu*)7697 *galU* *galK*  $\lambda$  *rpsL* *nupG*). Transformed cells are plated on to LB agar 15 mg/L tetracycline and 3 g/L glucose. Liquid cultures are grown in LB broth containing 15 mg/L tetracycline and 3 g/L glucose and plasmid DNA is isolated from these cultures using standard miniprep methods. Plasmid DNAs are analyzed by restriction analysis for correct integration of the *tet* fragment into the pUC19 backbone. Since integration of the fragment was unspecific with respect to orientation, only about 50% of all insert containing clone had the fragment inserted in the correct orientation, i.e. the *tet* gene

running in the same direction as the the amp gene in pUC19. A larger amount of DNA is isolated from liquid cultures of a few clones and subjected to more detailed restriction analyses. Of those clones showing correct restriction patterns, one is selected for further cloning work.

The respective plasmid was designated pMG402. It is identical to pUC19 in all features and functions but for the fact that it must be grown on/in tetracycline-containing media instead of ampicillin-containing media. This way a *tet* resistant high copy vector suitable for industrial purposes is generated.

Features of plasmid pMG402:

bps 1954-680: pUC19 backbone (= pUC19 lacking the *amp* promoter and structural gene)  
bps 681-1953: *tet* promoter and structural gene from pBR322

rhIFN $\alpha$ 2b is expressed as an N-terminal fusion with the signal sequence of glutaryl 7-ACA acylase from *Pseudomonas diminuta* CCM 3987 (*gac1ss* = SEQ ID NO 5) directing the protein to the periplasm with concurrent processing (cleaving off) of the signal sequence by the host cell's signal peptidase apparatus.

Amino acid sequence of *gac1ss* (27 aa): MLRVLHRAAS ALVMATVIGL APAVAFA

In the 3' region of the coding sequence of the *gac1ss* a *Sac* II restriction endonuclease site is introduced via the 3' PCR primer creating a silent mutation (amino acid sequence unchanged). This *Sac* II site allows fusion of the *gac1ss* coding region with the rhIFN $\alpha$ 2b gene.

The structural gene for rhIFN $\alpha$ 2b is synthesized chemically. It differs from the natural human cDNA sequence in 48 of 165 codons and is designed to eliminate any weak and error prone codons.

In the following table, codon changes are indicated. In the table, "Natural codon" refers to the cDNA sequence published by Streuli et al., 1980 (GenBank Accession Number V00548). The amino acid numbers refer to mature hIFN $\alpha$ 2b (starting with Cys 1). The amino acid

sequence to be encoded by the synthetic gene is taken from the SwissProt Database, Accession number P01563/P01564 (amino acids 24 to 188).

Exchange no.	Amino acid	Natural codon	Synthetic codon	Exchange no.	Amino acid	Natural codon	Synthetic codon
1	Cys 1	TGT	TGC	25	Leu 80	CTC	CTG
2	Pro 4	CCT	CCG	26	Leu 81	CTA	CTT
3	Arg 12	AGG	CGG	27	Leu 88	CTC	CTG
4	Arg 13	AGG	CGA	28	Asn 93	AAT	AAC
5	Leu 17	CTC	CTT	29	Cys 98	TGT	TGC
6	Arg 22	AGG	CGG	30	Ile 100	ATA	ATC
7	Arg 23	AGA	CGA	31	Gly 102	GGG	GGT
8	Ser 28	TCC	TCT	32	Gly 104	GGG	GGT
9	Leu 30	TTG	TTA	33	Thr 106	ACA	ACT
10	Asp 32	GAC	GAT	34	Pro 109	CCC	CCG
11	Arg 33	AGA	CGA	35	Ser 115	TCC	TCT
12	Phe 36	TTT	TTC	36	Arg 120	AGG	CGA
13	Gly 37	GGA	GGT	37	Arg 125	AGA	CGG
14	Phe 38	TTT	TTC	38	Leu 128	CTC	CTG
15	Pro 39	CCC	CCG	39	Pro 137	CCT	CCG
16	Phe 43	TTT	TTC	40	Cys 138	TGT	TGC
17	Gly 44	GGC	GGT	41	Arg 144	AGA	CGA
18	Pro 54	CCT	CCG	42	Arg 149	AGA	CGG
19	Val 55	GTC	GTA	43	Phe 151	TTT	TTC
20	Leu 56	CTC	TTG	44	Ser 154	TCA	TCT
21	Asn 65	AAT	AAC	45	Thr 155	ACA	ACC
22	Leu 66	CTC	CTG	46	Ser 160	AGT	TCT
23	Thr 69	ACA	ACT	47	Arg 162	AGA	CGA
24	Ser 72	TCA	TCT	48	Ser 163	AGT	AGC

The resulting gene allows efficient and precise transcription and translation of rhIFN $\alpha$ 2b in *Escherichia coli*. Since the gene is designed for expression in a bacterial system it does not contain any untranslated sequences (introns etc.).

The structural gene is chemically synthesized. In brief, overlapping complementary oligonucleotides about 30 to 50 nucleotides in length are synthesized in a way to cover both strands of the structural gene sequence without any gaps. The oligonucleotides are hybridized to each other and ligated using T4 DNA Ligase. The reaction product is cut with restriction endonucleases and cloned into the pUC18 vector. The resulting plasmid is sequenced and shows the correct sequence.

The synthetic gene on this plasmid does not contain the *gac* signal sequence. This part of the coding region is introduced via the *gac* fragment containing promoter, RBS and signal sequence and fused to the *rhIFN $\alpha$ 2b* structural gene.

The *gac* fragment is generated by chemical synthesis. For example, overlapping complementary oligonucleotides about 30 to 50 nucleotides in length are synthesized in a way to cover the full length of both strands of the *gac* fragment (including the restriction endonuclease recognition sites on both sides plus a minimum of 6 additional basepairs to allow efficient cleavage) without any gaps. The oligonucleotides are then hybridized to each other (e.g. by heating and subsequent cooling) and ligated using T4 DNA Ligase. The reaction product is then cut with the respective restriction endonucleases (*Xba*I and *Eco*R I) and cloned into the pMG402 vector (see below).

In the alternative, the *gac* fragment containing promoter, RBS and signal sequence can be amplified from a plasmid comprising such elements like plasmid pKS55, which construction is described in CS patent No. 278,515. The *gac* gene cloned therein has been derived from a strain of *Pseudomonas diminuta* (CCM 3987). Amplification is carried out using a high fidelity PCR system. The restriction endonuclease sites needed for cloning are introduced via the following PCR primers.

Primers:

1. 5'-Phosphate - GGGGGTCTAGACCAACAA $\overline{\text{C}}$ ATCTTCAACGTCTACC -3'  
(SEQ ID NO 6)

2. 5'-Phosphate - CC CCC CGA ATT CAC TAG TAC GCG TCT CTC TCC -3'  
(SEQ ID NO 7)

There will be no difference in performance between a *gac* fragment generated via high fidelity PCR amplification and a *gac* fragment generated by chemical synthesis.

The thus created *gac* fragment has the following nucleotide sequence (SEQ ID NO 8):

5' -GGGGGGTCTAGACCAACAACATCTTCAACGTCTACCCGACCAAGATTGAGAGCCGTCGG  
CCGACCTGGGCAATGGGATGTACAGCGGGCTTGCGCGTTCACCGGCGGATCCT  
GGTTCGTACGCGCCGCTACAAGTGGTGATCTAGGGGAACGTTCCGGGGGCGTCGTGCA  
ACGGCGTCTCCGGATCTGGGTGAGAGGGGAAATCCATGCTGAGAGTTCTGCACCGGGCGG  
CGTCCGCTTGTTTATGGCGACTGTGATCGGCCTTGCGCCCGCGGAGAGACGCGTACT  
AGTGAATTCGGGGGG-3'

The *gac* fragment (either synthetic or created via PCR) and the vector plasmid pMG402 are ligated using the *Xba* I and *Eco*R I sites. This way the expression vector pMG412 is generated.

The expression vector, pMG412, contains codons 1 - 23 + the first nucleotide of codon 24 of the *gac* signal sequence. Into codons 22-24 the *Sac* II site is introduced by silent mutation. Anything downstream of the *Sac* II site in pMG412 is primer or vector sequence.

The last two nt of codon 24 + codons 25-27 are introduced by the forward PCR primer for the target structural gene (*rhIFN $\alpha$ 2B*, see above). Such a primer therefore contains the following elements:

Cutting overhang (e.g. 6 nucleotides) - *Sac* II site - tc gcc ttt gcg (SEQ ID NO 9)- hybridizing region corresponding to the 5' end of the "mature" target gene.

In particular, a suitable primer has the following nucleotide sequence (SEQ ID NO 10):

TT GCG CCC GCG GTC GCC TTT GCG - hybridizing region (*Sac* II underlined)

The last amino acids (24-27) of the *gac* signal sequence are V A F A (SEQ ID NO 11).

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From the plasmid construct described above the rhIFN $\alpha$ 2b gene is amplified using a high fidelity PCR system. The 5' PCR primer contains the *Sac* II site for fusing the gene with the *gac* fragment plus the last four codons of the *gac* signal sequence. The 3' primer contains the TAA (ochre) stop codon and the *Mlu* I site for cloning. The amplification of the Interferon alpha structural gene generates the following fragment (SEQ ID NO 12):

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GGGGGGCCGCGGTCGCCTTTGCGTGCGATCTGCCGCAAACCCACAGCCTGGGTAGCCGGC
GAACCTTGATGCTTCTGGCACAGATGCGGCGAATCTCTCTTTCTCTTGCTTAAAGGATC
GACATGACTTCGGTTTCCCGCAGGAGGAGTTCGGTAACCAAGTCCAAAAGGCTGAAACCA
TCCCGGTATTGCATGAGATGATCCAGCAGATCTTCAACCTGTTCACTGTAAGGACTCTT
CTGCTGCTTGGGATGAGACCTGCTTGACAAATTTACACTGAACTGTACCAGCAGCTGA
ACGACCTGGAAGCCTGCGTGATCCAGGGTGTGGGTGTGACTGAGACTCCGCTGATGAAGG
AGGACTCTATTCTGGCTGTGCGAAAATACTTCCAACGGATCACTCTGTATCTGAAAGAGA
AGAAATACAGCCCGTGCCTGGGAGGTTGTCCGAGCAGAAATCATGCGGTCTTTCTCTT
TGCTTACCAACTGCAAGAACTTTACGAAGCAAGGAATAATACGCGTGAATTCGGGGGG
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This rhIFN $\alpha$ 2b PCR fragment and pMG412 are ligated using the *Sac* II and *Mlu* I sites. This way the final production/expression plasmid pMG414 was generated. Both strands of pMG414 are sequenced and show no differences to the expected sequence.

Features of plasmid pMG414 (total size 3668 bps):

- bps 2728-256: pUC19 backbone, part 1
- bps 257-546: *gac* fragment (promoter, RBS, signal sequence)
- bps 547-1044: synthetic rhIFN $\alpha$ 2b gene (including TAA stop)
- bps 1045-1454: cloning sites + pUC19 backbone, part 2
- bps 1455-2727: *tet* gene from pBR322 (promoter/RBS 1455-1536, coding sequence including TAA stop 1537-2727)

Thereby, the *gac* fragment containing promoter, RBS and signal sequence is fused to the rhIFN $\alpha$ 2b structural gene - using a restriction endonuclease site at the 3' end of the *gac* fragment introduced by a PCR primer. The same site is fused to the 5' end of the rhIFN $\alpha$ 2b structural gene, also by the way of a PCR primer. So after cloning both elements (*gac* fragment and rhIFN $\alpha$ 2b structural gene) into the basic vector a gene encoding a *gac*1ss-

rhIFN $\alpha$ 2b fusion protein is generated. Of its total 192 codons (576 nucleotides) the first 27 encode the *gac* signal sequence not present in the final protein and amino acids 28 to 192 encode mature rhIFN $\alpha$ 2b (165 amino acids, cysteine 1 to glutamic acid 165)

The nucleotide sequence of the expression cassette used in the rhIFN $\alpha$ 2b expression plasmid pMG414 (807 bps) (see below) and amino acid sequence of the *gac*1ss-rhIFN $\alpha$ 2b fusion protein is shown as follows (SEQ ID NO 13):

5'-TCTAGACCAACAACATCTTCAACGCTACCCGACCAAGATTCAGGAGCCGTCGGCCGACC  
TGGGCAATGGGATGTACAGCGGGCTTGC GCCGTTTCGGCTTCACCGGCGGATCCTGGTTCCG  
TACGCGCCGCTACAAGTGGTGATCTAGGGGAACGTTCCGGGGGCGTCGCTGCAACGGCG  
TCTCCGGATCTGGGTGAGAGGGGAAATCC

ATG CTG AGA GTT CTG CAC CGG GCG GCG TCC GCC TTG GTT ATG GCG  
M L R V L H R A A S A L V M A

ACT GTG ATC GGC CTT GCG CCC GCG GTC GCC TTT GCG  
T V I G L A P A V A F A

TGC GAT CTG CCG CAA ACC CAC AGC CTG GGT AGC CGG CGA ACC TTG  
C D L P Q T H S L G S R R T L

ATG CTT CTG GCA CAG ATG CGG CGA ATC TCT CTT TTC TCT TGC TTA  
M L L A Q M R R I S L F S C L

AAG GAT CGA CAT GAC TTC GGT TTC CCG CAG GAG GAG TTC GGT AAC  
K D R H D F G F P Q E E F G N

CAG TTC CAA AAG GCT GAA ACC ATC CCG GTA TTG CAT GAG ATG ATC  
Q F Q K A E T I P V L H E M I

CAG CAG ATC TTC AAC CTG TTC AGC ACT AAG GAC TCT TCT GCT GCT  
Q Q I F N L F S T K D S S A A

TGG GAT GAG ACC CTG CTT GAC AAA TTC TAC ACT GAA CTG TAC CAG  
W D E T L L D K F Y T E L Y Q

CAG CTG AAC GAC CTG GAA GCC TGC GTG ATC CAG GGT GTG GGT GTG  
Q L N D L E A C V I Q G V G V

ACT GAG ACT CCG CTG ATG AAG GAG GAC TCT ATT CTG GCT GTG CGA  
T E T P L M K E D S I L A V R



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AAA TAC TTC CAA CGG ATC ACT CTG TAT CTG AAA GAG AAG AAA TAC
K   Y   F   Q   R   I   T   L   Y   L   K   E   K   K   Y

AGC CCG TGC GCC TGG GAG GTT GTC CGA GCA GAA ATC ATG CGG TCT
S   P   C   A   W   E   V   V   R   A   E   I   M   R   S

TTC TCT TTG TCT ACC AAC TTG CAA GAA TCT TTA CGA AGC AAG GAA
F   S   L   S   T   N   L   Q   E   S   L   R   S   K   E

```

**TAA**

TACGCGTACTAGTGAATTC-3'

The sequence as shown is divided into sub-paragraphs / regions which comprise:

1. the *gac* promoter and RBS (first paragraph, bps 257 to 465 of pMG414, see below),
2. the *gac* signal sequence coding region (second paragraph, bps 466 to 546 of pMG414, see below),
3. the synthetic gene for rhlFN $\alpha$ 2b (third paragraph, bps 547 to 1044 of pMG414 (see below) – including the TAA stop codon), and
4. the 3' cloning linker (fourth paragraph, bps 1045 to 1063 of pMG414, see below).

On the pMG414 these four regions are directly joined to one another. They are separated in the figure for reasons of lucidity only.

The start (ATG) and the stop (TAA) codons of the open reading frame are shown in bold.

The first (TGC) and the last (GAA) codon of mature rhlFN $\alpha$ 2b are underlined.

The restriction endonuclease sites used for cloning are boxed. These are:

- *Xba* I (TCTAGA) and *Eco* R I (GAATTC) for the introduction of the *gac* fragment (promoter, RBS, signal sequence, *Sac* II, *Mlu* I, *Spe* I sites)
- *Sac* II (CCGCGG) and *Mlu* I (ACGCGT) for the introduction of the rhlFN $\alpha$ 2b PCR fragment (including four codons for the last four amino acids of the *gac*1ss, the 495 bp synthetic gene for mature rhlFN $\alpha$ 2b, and the TAA(T) stop codon).

The *gac* promoter shows high constitutive / basal activity, the addition of a chemical inducer or a physical stimulus (change in culture conditions) is not required.

### 1.3 Cloning and establishment of the recombinant cell line

The expression plasmid pMG414 is introduced into the host strain ATCC PTA-3132 (=W3110 (ATCC 27325)) by electroporation. Electrocompetent cells are prepared according to a standard protocol, electroporation is carried out in 0.1 mm cuvettes at 1800 V using an Eppendorf Electroporator 2510.

After electroporation the reaction is suspended in liquid medium and plated onto agar plates containing tetracycline.

Starting point for selection of a suitable cell clone is a thus obtained transformation plate. Various clones from this plate are grown in liquid culture and cryopreserved as research cell banks. Their productivity is tested in shake flask experiments and compared. The best clone (E1/116) is used for further development.

The best clone may show good productivity but relatively poor growth. This poor growth can result from various factors, e.g. product toxicity to the host cell, metabolic burden due to product synthesis etc. The addition of glucose often brings some improvement because glucose downregulates (e.g. by catabolite repression) many promoters used for recombinant protein expression. Also, glucose has a general positive effect on the growth of *E. coli* because it can be directly introduced into the metabolism as a carbon source.

In the case of E1/116, a clear positive effect of glucose on growth is observed. The best results are achieved with glucose concentrations between 2 and 5 g/L. To adapt the cell line to cope with product formation and consequently to better growth in the absence of glucose, the strain is therefore grown in liquid medium in shake flasks for several passages ("shake flask cascade").

More specifically, a cryovial of E1/116 is thawed and the cell suspension streaked onto glucose free LB agar plates containing tetracycline. The plate is incubated at 37°C until the colonies reach a sufficient size for inoculating a liquid culture. Colonies are transferred from

the plate into small shake flasks filled with 15 mL of glucose free LB broth containing tetracycline. The cultures are shaken at 37°C until they reach an optical density at 600 nm of above 0.5 (typically > 1.0). For this first round this takes up to 48 hours due to the poor growth characteristics of the original isolate.

The procedure described in the above paragraph is performed five consecutive times with the liquid culture of the previous round being streaked onto plates and the colonies from the plates serving to inoculate the next liquid culture. From each liquid culture optical density is determined and a sample was taken for determination of product titer using SDS-PAGE – Western Blot. Clones from the culture with the best combination of growth and productivity are the used to initiate the next round.

In the course of the different rounds of this culture cascade (i.e. multiple propagation and reisolation steps) the growth characteristics of the (sub)strain(s) gradually improve. By choosing the strain with the best combination of growth and productivity in each round, titers are also gradually increased. After the fifth round again single colonies are generated on LB agar plates containing tetracycline and used to inoculate a liquid culture containing tetracycline for the generation of a primary seed lot (PSL). The culture is grown at 37°C to an optical density of about 1.5, mixed with an equal amount of sterile 40% w/v glycerol, aliquoted into cryogenic vials and frozen at -80°C. This PSL is used as a starting point for the generation of the GMP cell banks (Master Cell Bank and Working Cell Bank) of the Interferon alpha 2b production strain. This reisolate is designated E1/116a. Reisolation processes like the one described above have proved to yield reproducible results.

E1/116a shows excellent growth characteristics in shake flasks and stirred bioreactors (fermenters). An inoculum suitable for starting a bioreactor can be grown in a shake flask starting from a Working Cell Bank vial in about 8 hours.

A Master Cell Bank is prepared under cGMP conditions from the primary seed lot described above. In brief, a PSL vial is thawed and plated onto tetracycline containing agar plates. A single colony is picked and used to inoculate the Master Cell Bank (MCB) shake flask culture (LB broth medium containing tetracycline). The cell suspension from the logarithmic growth phase is mixed 1+1 with 40 % w/v Glycerol, aliquoted at 1.8 mL into cryogenic vials, sealed in cryogenic tubing, and frozen in the liquid phase of a liquid nitrogen tank.

The Working Cell Bank is generated in the same way as the Master Cell Bank except that the shake flask culture is inoculated with cell suspension from a thawed MCB vial.

**Example 2: Fermentation process for production of recombinant human Interferon  $\alpha$  2B (rhIFN $\alpha$ 2B)**

The fermentation process is started by growing the strain E.coli K-12 W3110 obtainable from the Working Cell Bank as described above in shake flask cultures in Luria Bertani (LB-) medium at 37°C with the addition of the antibiotic tetracyclin hydrochloride to avoid growth of non-plasmid carrying cells.

The shake flask culture is then used to inoculate the seed culture (= pre-culture) medium (inoculum size = 0,4%). The medium for this pre-culture cultivation is based on deionized water containing glucose as a sole carbon source and yeast autolysate as a complex nitrogen source. In addition, anorganic salts like  $\text{KH}_2\text{PO}_4$ ,  $\text{K}_2\text{HPO}_4$ ,  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  are added to the medium. As an antifoam agent polypropylene glycole 2000 (PPG2000) is used. In particular, the pre-culture medium has the following composition:

**Pre-culture Basal Medium:**

Component	Amount
De-ionized water (WBI)	30 l
Yeast autolysate, KAT, Ohly	21.7 g/l
Glucose Monohydrate, pure	25.0 g/l
Ammonium Sulfate, p.a.	1.0 g/l
Potassium Phosphate Monobasic, p.a	1.5 g/l
Potassium Phosphate Dibasic, anhydrous, pure	3.0 g/l
Magnesium Sulfate Heptahydrate, p.a.	0.5 g/l
Polypropylene Glycole 2000	0.5 ml/l

These media components are sterilized together for 20 minutes at 121 °C. After cooling of the basal medium, an aliquot of a 5 g/L sterile stock-solution of the antibiotic Tetracycline

Hydrochloride is added to the basal medium (sterilization is performed by filtration (0.22  $\mu$ m filter)).

Stock-solution Tetracycline Hydrochloride (5 g/L):

Component	Amount
Tetracycline Hydrochloride cryst., Ph.Eur.	15 mg/l
De-ionized water (WBI)	

The cultivation-time for seed culture is about 16 hours. During cultivation of the seed culture pH-value is controlled to a set-point of  $7.0 \pm 0.2$  with sulfuric acid and sodium hydroxide or concentrated ammonia solution. Concentration of dissolved oxygen is kept at levels higher than 20% of saturation by increasing the stirrer speed. Stirrer speed at the beginning of the cultivation is set to 300 rpm, back-pressure in the vessel to 0,3 bar and aeration rate is controlled to 30 L/min (equivalent to "1 vvm"). Temperature is kept constantly at 37°C during cultivation. As a transfer criterion of broth to the main stage of the fermentation process, an increase of the dissolved oxygen concentration after consumption of the carbon source is used.

For main culture cultivation a medium based on deionized water, glucose as a carbon source and yeast autolysate as a complex nitrogen source is used. Besides the addition of the anorganic salts  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , PPG 2000 is used as an antifoam agent. The initial glucose is sterilized separatly and added to the sterile rest of the medium. Inoculum size to the main fermenter medium was in a range between 0,75 and 3%. In particular, the main culture medium has the following composition:

Main Culture Basal Medium:

Component	Amount
De-ionized water (WBI)	60 l
Yeast autolysate, KAT, Ohly	43.5 g/l
Ammonium Sulfate, p.a.	1.0 g/l
Calcium Chloride Dihydrate cryst., p.a.	0.3 g/l
Magnesium Sulfate Heptahydrate, p.a.	1.0 g/l

Component	Amount
Polypropylene Glycole 2000	0.5 ml/l

These media components are sterilized together for 20 minutes at 121 °C. After cooling, an aliquot of a 800 g/L separately heat-sterilized glucose stock-solution is added to the main culture basal medium (sterilization is performed for more than 30 minutes at 120 °C).

Glucose Stock-solution, 800 g/l:

Component	Amount
Glucose Syrup	12.5 ml/l
De-ionized water (WBI)	

The most important point during this cultivation is the necessity of a complete consumption of the initial glucose present in the medium. This leads to a sharp increase of dissolved oxygen concentration after about 9 hours of growth. By starting glucose feeding before total consumption of the initial glucose, no product formation is observed. Glucose limitation controlled by the feeding of the glucose-solution at a constant rate is therefore very important. The temperature during cultivation is controlled to a constant value of about 28°C. The initial stirrer speed is set to 300 rpm, the aeration rate is controlled to 100 L/min (equivalent to "1 vvm") and the back-pressure in the vessel is set to 0,3 bar. The pH-value is controlled to  $7,1 \pm 0,3$  with sulfuric acid and sodium hydroxide or concentrated ammonia solution. A peak of the pH-value up to 8.0 after consumption of the initially supplied glucose is acceptable.

The concentration of the dissolved oxygen is controlled to values higher than 20% of saturation. Dependent on the oxygen transfer capacity of the bioreactor DO-concentration is kept at levels higher than 20% of saturation, preferably between about 40 % and 100% of saturation, by first increasing the stirrer speed to a maximum value. If this is not sufficient, first aeration rate and after that back-pressure is increased, respectively. After a cultivation time between 48 and 192 hours (linear increase of product formation is observed with cultivation time) the culture is harvested and cooled to  $15 \pm 5$  °C and conditioned for downstream processing by the addition of sucrose/EDTA to the cooled broth.

The results of a fermentation batch is analysed based on the Westernblot technique or on HPLC-measurements after laboratory or pilot plant periplasmatic extraction of the product.

#### Example 3: Cell disruption and extraction

A fermentation broth containing host cells comprising the expressed interferon alpha 2B in the periplasmic space is adjusted with sulfuric acid to pH of  $5.0 \pm 0.1$  immediately after the fermentation and cooled down to  $4^{\circ}\text{C} \pm 2^{\circ}\text{C}$ . The low pH and the low temperature help to inactivate endogenous proteases.

The fermentation broth is adjusted to  $10^{\circ}\text{C}$  to  $20^{\circ}\text{C}$ , then without any concentration or washing of the cells, solid or liquid sucrose (200g sucrose/kg fermentation broth) and EDTA (concentration 10mM) are added and the pH adjusted to 8.0. After a selective one-step cell permeation protocol using osmotic shock (1+3 dilutions) with cooled water, whereby the fermentation broth comprising sucrose and EDTA is poured or pumped into the cooled (temperature about  $4^{\circ}\text{C}$ ) water, the released periplasmic extract is clarified. Polyethyleneimine is added to a final concentration of 0,05 % and the pH is adjusted to about 7,5 with acetic acid. After 15 to 45 minutes cell debris and DNA flocculate, leaving a clear crude extract containing interferon which may be subject to centrifugation to improve clarity.

This procedure leads to a clear periplasmic extract comprising the desired interferon alpha 2B in high yield with a purity of  $> 20\%$  with respect to the total protein content. Polyethyleneimine helps to separate the cell debris from the soluble protein extract leading to a very pure interferon solution.

#### Example 4: Chromatographic purification of recombinant human interferon $\alpha$ 2B (rhIFN $\alpha$ 2B)

##### 4.1 Capture by Cation Exchange Chromatography (CEX)

After pH adjustment to 4.8 – 5.2 with acetic acid and a filtration step using a 0.3 micron filter, the crude extract of Example 3 is applied to the CEX column (S ceramic HyperD F (Biosepra)). After a washing step with an equilibration buffer (20mM sodium acetate and 70mM NaCl at pH 5.0) the interferon is eluted with a step gradient at 175mM NaCl. The fraction collected is immediately processed by the process step of Example 4.2.

#### 4.2 Anion Exchange (AEX) Chromatography

The fraction from Example 4.1 is adjusted to a pH of 7.3 to 7.7 with sodium hydroxide, diluted and purified with water to a conductivity of 3.5 to 4.5 mS/cm and applied to the AEX column (Q ceramic HyperD F (Biosepra)). After washing, the interferon is eluted with a linear salt gradient (0-300mM NaCl) at about 150mM NaCl. Fractions are collected that have a purity of greater than or equal to 90 area % according to IPC reversed-phase HPLC and used directly in the next step (see Example 4.3).

#### 4.3 Hydrophobic Interaction Chromatography (HIC)

The fraction of Example 4.2 is diluted (1:1) with a stock solution of sodium sulphate (0.5% sodium sulphate), adjusted to pH 7.3 to 7.7 with NaOH or HCl and applied to the HIC column (Source 15PHE (Pharmacia)). After washing, the interferon fraction of Example 3 is eluted with a linear sodium sulphate concentration (800 – 0 mM sodium sulphate) at about 400mM sodium sulphate. The fractions collected that have a purity of greater than or equal to 93 area % and no impurity greater than or equal to 3% according to IPC reversed-phase HPLC are used directly in the next purification step.

#### 4.4 Cation Exchange Chromatography (CEX)

The collected fractions of Example 4 are diluted with water to a final conductivity of 7.5 to 8.5 mS/cm, adjusted to pH 4.3 to 4.7 with 99 to 100% acetic acid and applied to the CEX column (Toyopearl SP-650 S (TosoHaas)). After a washing step, the interferon is eluted in a linear NaCl gradient (0 – 300 mM NaCl) at about 250mM NaCl. The fractions are collected that have a purity of greater than or equal to 95 area % and no impurity greater than or equal to 3% according to IPC reversed-phase HPLC and are used directly in the next purification step.



#### 4.5 Size Exclusion Chromatography

The last purification step is a gel filtration step to remove dimers and other aggregates and to perform a buffer exchange for the final formulation. The Superdex 75 pg used in this step shows a good resolution even at a high load volume (5 % - 15 %). The SEC is performed in 25mM sodium phosphate and 130mM NaCl + 0.3mM EDTA at a pH of about 7.3 to 7.7.

The fractions with the highest purity (> 95 % main peak in RP-HPLC and no side peak > 3 %) are pooled to give the final bulk solution comprising the desired recombinant human interferon  $\alpha$  2B in pure form in a high yield.

#### Deposition of Microorganisms:

*E. coli* strain W3110 (ATCC 27325), as used herein, has been deposited with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209, USA on February 28, 2001, under the Designation No. PTA-3132.